CHAPTER III

MATERIALS AND METHODS

Cell Culture

The rat neuroblastoma N1E-115 cell line, a clone from C1300 (American Type Culture Collection, Manassas, VA, USA) were used in these experiments.

Chemicals

- 90% Asiaticoside (Changzhou Natural Products Development, China) with the following specifications.

Test Item	Standard	Assay Result
Color	White powder	White powder
TLC	Conforms	Conforms
Loss on drying	≤ 5%	1.39%
Heavy metals	< 10 ppm	< 10 ppm
Sulphated ash	< 0.5%	0.36%
Assay (HPLC)	Asiaticoside 90%	Asiaticoside 90.03%
Granule size	98% pass 100 mesh	98% pass 100 mesh
Total bacteria count	Less than 1000/gm	121/gm
Yeast/moulds	Less than 100/gm	Negative
E. coli	Negative	Negative
Pseudomonas aeruginosa	Negative	Negative
Staphylococcus aureus	Negative	Negative

- *Centella asiatica* extract (37.20% Madecassoside and 38.56% Asiaticoside) (kindly provided by Associate Professor Dr. Ekarin Saifa, Department of Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University)
- 5-5'-dithiobis-(2-nitrobenzoic acid) [Sigma, U.S.A.]
- Dimethyl sulfoxide (DMSO) [BDH, England]
- Dulbecco's Modified Eagle's Medium (DMEM) [Gibco, U.S.A.]
- Dulbecco's Phosphate Buffered Saline (DPBS) [Sigma, U.S.A.]

- Ethylenediaminetetraacetic acid (EDTA) [Sigma, U.S.A.]
- Fetal bovine serum (FBS) [Hyclone, U.S.A.]
- Glutathione (reduced form) (GSH) [Sigma, U.S.A.]
- Glutathione reductase (GRx) [Sigma, U.S.A.]
- Griess reagent (modified) [Sigma, U.S.A.]
- Human transferrin [Sigma, U.S.A.]
- In vitro toxicology assay kit (Lactate Dehydrogenase Based) [Sigma, U.S.A.]
- Insulin from bovine pancrease [Sigma, U.S.A.]
- MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5diphenyltetrazolium bromide) [Sigma, U.S.A.]
- β-Nicotinamide adenine dinucleotide phosphate, reduced form (β-NADPH)[Sigma, U.S.A.]
- Penicillin G sodium [Sigma, U.S.A.]
- Progesterone [Sigma, U.S.A.]
- Putrescin dihydrochloride [Sigma, U.S.A.]
- S-nitroso-N-Acetylpenicillamine (SNAP) [Sigma, U.S.A.]
- Sodium nitrite [Sigma, U.S.A.]
- Streptomycin sulfate [Sigma, U.S.A.]
- Sulfosalicylic acid [Sigma, U.S.A.]
- Triton X-100 [Sigma, U.S.A.]
- Trypan blue [Sigma, U.S.A.]
- Trypsin [Sigma, U.S.A.]

Materials and Instruments

- 24-well cell culture plate [Nunc, Denmark]
- 96-well microtiter plate [Nunc, Denmark]
- Cell culture flask: 25 cm^2, 75 cm^2 [Nunc, Denmark]
- Conical tube: 15 ml, 50 ml [Nunc, Denmark]
- Sterile Millex – GV (0.22μm filter unit) [Millipore, U.S.A.]
- Autoclave [Hirayama, Japan]
- Carbon dioxide incubator [Forma Scientific, U.S.A.]
- Centrifuge [Kokusan, Japan]
- Hemocytometer (Depth 0.100 mm) [Improved Neubauer]
- Inverted microscope, Axiovent 135 [Zeiss, Germany]
- Laminar air flow hood [Hepaco, U.S.A.]
- Microliter pipette : 10-100 μl [Labsystems, Finland]
- Microliter pipette : 200-1000 μl, 1-5 ml [Labsystems, Finland]
- Microplate reader [Anthos htl, Australia]
- Microplate reader [Perkin Elmer, Germany]
- pH meter [Beckman Instruments, U.S.A.]
- Sonicator [Elma, Germany]
- Vortex mixer [Clay adams, U.S.A.]
- Water bath [Thelco, U.S.A.]

Preparation of Asiaticoside, *Centella asiatica* Extract and SNAP Solutions

In all experiments, asiaticoside and *Centella asiatica* extract were dissolved in double distilled water and SNAP was dissolved in DMSO for the addition into cell culture medium. The final concentration of DMSO in the culture medium was ≤ 0.5%. Control groups were treated with the corresponding amount of their respective solvents.

Preparation of Culture Media

Dulbecco's modified Eagle's medium (DMEM, high glucose) was supplemented with 3.7 mg/ml sodium bicarbonate, 100 units/ml penicillin G sodium and 100 μg/ml streptomycin sulfate, 10% (v/v) fetal bovine serum (FBS). Serum-free DMEM was composed of normal DMEM supplemented with 30 nM selenium, 30 μg/ml human transferrin, 5 μg/ml insulin, 100 μM putrescin and 20 nM progesterone.

Preparation of Neuroblastoma Cell Cultures

The rat neuroblastoma cell line, clone N1E-115 (passage numbers 21-27), was obtained from American Type Culture Collection. The cell suspension was plated into 25 cm^2 plastic culture flasks and grown in DMEM containing 10% FBS in a humidified incubator maintaining at 37 °C and containing 5%CO_2-95% air atmosphere. When the cultures reached confluency, the cells were removed from the flasks by trypsinization. The culture medium was aspirated and further incubated with 0.25% trypsin-EDTA solution for 3 minutes at room temperature, followed by the addition of an equal volume of DMEM containing 10% FBS. The cell suspension was centrifuged at 1,250 rpm for 4 minutes at 4°C and the pellet was suspended in fresh DMEM containing 10%FBS. The cells were counted on hemocytometer using trypan blue exclusion. After dilution with culture medium, aliquots of 1 ml were plated at a density of 5×10^3 cells/cm^2 in 24-well culture plates and cultures were incubated in a humidified atmosphere of 5% CO_2-95% air at 37 °C. The medium will be changed every 2-3 days and subcultured once they reached 80-90% confluence. After growth to confluency [approximately 48 hours after plating], the cells were used in the experiments.

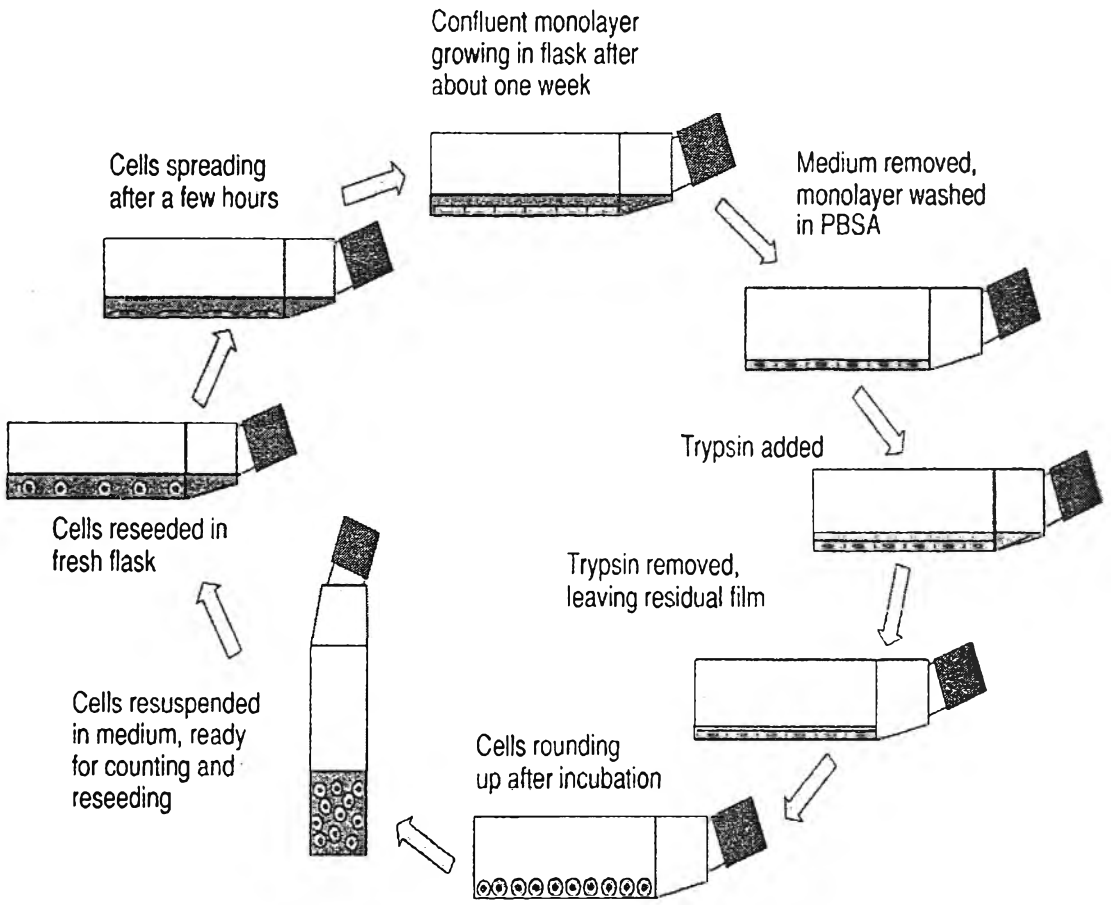

Fig. 5 Subculture method (Freshney, 2000)

Experimental Conditions

1. Treatment of cultured N1E-115 cells with asiaticoside

Cultures were treated with 1, 10, 25, 50, 75 and 100 μM of asiaticoside for 24 and 48 hr in serum-free, DMEM containing N2 media supplements.

2. Treatment of cultured N1E-115 cells with *Centella asiatica* extract

Cultures were treated with 1, 10, 25, 50, 75 and 100 μg/ml of *Centella asiatica* extract for 24 and 48 hr in serum-free, DMEM containing N2 media supplements.

3. Exposure of cultured N1E-115 cells to SNAP

Cultures were exposed to SNAP at final concentrations of 0.25, 0.5, 1, 1.5, 2 and 3 mM in serum-free, DMEM containing N2 media supplements for 6, 12 and 24 hr.

4. Treatment of SNAP-exposed N1E-115 cell cultures with test agents

4.1 Pre-treatment with asiaticoside

Cultures were first incubated with 1, 10, 25, 50, 75 and 100 μM of asiaticoside in serum-free, DMEM containing N2 media supplements for 24 and 48 hr. At the indicated time, the medium culture was replaced with fresh serum-free, N2-supplemented DMEM containing 1 mM of SNAP and further incubated for 24 hr.

4.2 Pre-treatment with *Centella asiatica* extract

Cultures were first incubated with 1, 10, 25, 50, 75 and 100 μg/ml of *Centella asiatica* extract in serum-free, DMEM containing N2 media supplements for 24 and 48 hr. At the indicated time, the medium of the culture was replaced with fresh serum-free, N2-supplemented DMEM containing 1 mM of SNAP and further incubated for 24 hr.

4.3 Co-treatment with asiaticoside

Cultures were incubated with asiaticoside at concentrations of 1, 10, 25, 50, 75 and 100 μM in combination with SNAP at a concentration of 1 mM in serum-free DMEM containing N2 media supplement for 24 hr of incubation.

4.4 Co-treatment with *Centella asiatica* extract

Cultures were incubated with *Centella asiatica* extract at concentrations of 1, 10, 25, 50, 75 and 100 μg/ml in combination with SNAP at a concentration

of 1 mM in serum-free DMEM containing N2 media supplement for 24 hr of incubation.

Analytical Methods

MTT reduction colorimetric assay

Cellular dehydrogenase activity, which is considered to reflect mitochodrial activity, was measured by the reduction of tetrazolium salt MTT [3-(4,5-dimethylthiazole-2-yl)-2,5 diphenyl tetrazolium bromide to formazan. Mitochondrial dehydrogenase in viable cells converts the soluble yellow form of the salt into an insoluble, intracellular purple formazan which is quantitated spectrophotometrically by solubilization in an organic solvent (Desagher, Glowinski, and Premont, 1996).

MTT reduction was analyzed by adding 15 μl of the MTT stock solution [5 mg/ml in phosphate-buffered saline (PBS)] to the medium in each well (final concentration was 100 μg/ml). Cultures were incubated in a CO_2 incubator at 37°C for 1 hr and the medium in each well was aspirated off without disturbing the formazan precipitate. Then 400 μl of DMSO was added to each well in order to solubilize the formazan crystals. Following through formazan solubilization , 100 μl aliquots of soluble fotmazan were transferred to a 96-well microtiter plate. The cellular reduction of MTT was determined by measuring the absorbance at 570/620 nm with a Anthos Labtec HT2 version 1.21E dual wavelength microplate reader. The results were expressed as percentage of MTT reduction relative to that of control culture.

Lactate dehydrogenase release assay

Released LDH is a biologically stable enzymatic marker that correlates linearly with cell death. Cell viability was determined by assaying the medium from each well for lactate dehydrogenase (LDH) activity using an in vitro toxicology assay kit [Sigma, U.S.A.] according to the manufacture's instructions. The assay is based on the reduction of NAD to $NADH/H^+$ by the action of LDH-catalyzed conversion of lactate to pyruvate. The reduced NAD (NADH) is then utilized in the conversion of a tetrazolium dye. The resulting colored compound is measured spectrophotometrically.

Briefly, medium LDH was assayed by pipetting 100 μl of culture medium from each well into a 96-well microtiter plate. Cellular LDH was measured by

carefully aspirated off the remaining culture medium and solubilized cells with 1 ml of 0.5% Triton X-100 in PBS after which 100 µl aliquots were pipetted into a 96-well microtiter plate. The reaction was started by adding 50 µl of lactate dehydrogenase assay mixture into each well. The plate was covered with an aluminium foil to protect from light and incubated at room temperature for 20-30 min. The reaction was terminated by the addition 50 µl of 1N HCl into each well. The light absorbance in each well was measured at 510/620 nm with a Anthos Labtec HT2 version 1.21E dual wavelength microplate reader. The total activity of LDH in control cultures was considered to represent 100% cell viability. The LDH release which reflects cell death was presented as percentage of total LDH activity by the following formula:

$$\%\text{LDH release} = \frac{\text{LDH activity in medium} \times 100}{\text{LDH activity in medium} + \text{LDH activity in cells}}$$

In most cases, comparative LDH release in test conditions was expresses as the percentage of that in control conditions.

Thiobarbituric acid reactive substances (TBARS) assay

The sensitivity of measuring Thiobarbituric Acid Reactive Substances (TBARS) as made this assay the method of choice for screening and monitoring lipid peroxidation, a major indicator of oxidative stress. The TBA test is a colorimetric technique, which measures the absorbance of the pink compound formed between TBA and TBARS.

TBARS was assayed by the following method. Briefly, The culture medium was aspirated off, then 300µl of 2% SDS was added in each well and incubated at room temperature for 30 min. 250 µl of the lysate of each well was pipetted into 15 ml conical tube. After that, 12.5 µl of 4% butylated hydroxytoluene in ethanol, 250 µl of 10% phosphotungstic acid in 0.5M sulfuric acid, and 125 µl of 0.7% thiobarbituric acid were added respectively. The mixture was incubated at 95°C on water bath for 60 min. The reaction was stopped by adding 500 µl of n-butanol for extracting TBARS. Bring the extract to centrifuge at 3,000 rpm for 5 min, then 200 µl of the supernatant

was collected for measuring at 485/435 nm by the fluorometer [Perkin Elmer, Wallac].

The average absorbance of each standard was plotted against its amount of TEP (5-400μM), which 1M TEP equal to 1M MDA. The best fit regression line was drawn through the points. The amount of MDA in each unknown sample was obtained by comparing its absorbance against the standard curve.

Glutathione assay

Cellular GSH levels were measured enzymatically by using a modification of the procedure of Tietze. This method is based on the determination of a chromophoric product, 2-nitro-5-thiobenzoic acid, resulting from the reaction of 5-5'-dithiobis-(2-nitrobenzoic acid) with GSH. In this reaction, GSH is oxidized to GSSG, which is then reconverted to GSH in the presence of GSH reductase (GRx) and NADPH. The rate of 2-nitro-5-thiobenzoic acid formation is followed at 450 nm (Tietze, 1969).

Cells were washed with ice-cold PBS and immediately collected by scraping with 0.5 ml of 1% (wt/vol) sulfosalicylic acid. Cell lysates were transferred to 1.5 ml Eppendorf tubes and centrifuged at 12,000 rpm for 5 min at 4°C. Following cell extraction, 20 μL aliquots of supernatant were transferred into 96-well microtiter plate and the volume was made up to 100μl with 0.1 M phosphate buffer containing 1 mM EDTA (pH 7.5). The reaction was started by adding 100 μl Of reaction mixture [0.15 mM 5,5'-dithio-bis (2-nitrobenzoic acid), 0.2 mM NADPH, and 1 U/ml glutathione reductase in 0.1 M sodium phosphate (pH 7.5)]. The formation of 2-nitro-5-benzoic was monitored at 10-s intervals for 15 cycles at 450/620 nm by using a microplate reader [Anthos htl]. The slope of the initial rate of reaction was used for calculating GSH content from a standard obtained by plotting known amount of GSH (1-100 μM). The cellular content of GSSG was typically less than 2% of the GSH level and was not considered.

Nitrite assay

The production of NO was assayed as nitrite accumulated in the culture medium by using a colorimetric reaction with Griess reagent, which converts nitrite into a colored azo compound. This method is based on the reaction that sulfanilic acid is quantitatively converted to a diazonium salt in the presence of nitrite in acid solution. The diazonium salt is then coupled to N-(1-naphthyl)ethylenediamine, forming an azo dye that can be quantitated spectrophotometrically (Grisham et al., 1996).

Briefly, 50 μl of the medium was mixed with an equal volume of the Griess reagent modified [Sigma, U.S.A.]. After 15-min at room temperature with protect from light, the absorbance at 510 nm was measured with a microtiter plate reader [Anthos htl]. The nitrite concentration was determined from a sodium nitrite standard curve.

Statistical analysis

All results were presented as the mean ± SEM values from 6 independent cultures, with duplicate replications in each experiment. Differences among means were analyzed using one-way analysis of variance (ANOVA). One-way ANOVA followed by Dunnett's test was used for planned multiple comparisons. All statistical analysis was performed by using a statistical program SPSS. A value of $P<0.05$ was considered to be significant.